Human Herpesvirus 6 Induces Interleukin-1β and Tumor Necrosis Factor Alpha, but Not Interleukin-6, in Peripheral Blood Mononuclear Cell Cultures

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The human herpesvirus 6 (HHV-6) is known to interact intimately with cells of the immune system. Here we report that HHV-6 is a potent inducer of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) in cultures of peripheral blood mononuclear cells. In contradistinction, HHV-6 has no effect on IL-6 synthesis. Maximal IL-1β and TNF-α gene transcription, as detected by polymerase chain reaction amplification analysis, is observed at 12 and 6 h postinfection, respectively. Release of IL-1β and TNF-α into the culture supernatants peaked at 24 h and gradually decreased with time. Heat-inactivated virus was unable to stimulate IL-1β and TNF-α syntheses, whereas UV-irradiated virus retained the full monokine-inducing potential of the native particle. Preincubation of viral preparation with neutralizing anti-HHV-6 antibody resulted in the abrogation of this cytokine-inducing effect, whereas treatment of cells with phosphonoacetic acid (an inhibitor of viral DNA polymerase activity) had no effect on the ability of the virus to stimulate monokine release. These results indicate that HHV-6 can exert a strong immunomodulatory effect by stimulating the cells of myeloid lineage to produce these cytokines.

The human herpesvirus 6 (HHV-6) is one of the most recently discovered members of the *Herpesviridae* family (28). It was first isolated from the cultures of peripheral blood leukocytes of patients with lymphoproliferative disorders and AIDS (28). The distinct nature of HHV-6 compared with other human herpesviruses was confirmed by molecular and immunological analyses (19). Like other human herpesviruses, HHV-6 is also ubiquitous and is mainly contracted during the first 3 to 5 years of life (5, 26, 29). The routes of transmission of the virus have not been fully identified, although the report of frequent shedding in the saliva suggests a horizontal mode of dissemination (18). HHV-6 is the presumed etiologic agent of the childhood disease exanthem subitum, also known as roseola infantum (32, 35). Moreover, HHV-6 is responsible for approximately 12% of heterophile-negative infectious mononucleosis cases (31). Since HHV-6 DNA fragments have been isolated from both salivary and parotid glands (15) and from some tumors of B-cell origin (6), the possibility of viral reactivation and subsequent pathologic abnormalities should not be overruled. This is supported by serological analyses which indicate an increase in anti-HHV-6 antibody titer in patients afflicted with lymphoproliferative disorders such as leukemia and lymphoma (8) as well as in immunocompromised patients such as those suffering from AIDS (23). Moreover, the apparent tropism of HHV-6 for cells of the immune system, namely, CD4+ T cells, B cells, monocytes/macrophages, and megakaryocytes, might lead to immunological disturbance following an infection (2, 24, 25).

Because of the intimate relationship between HHV-6 and cells of the immune system, it was of interest to investigate the effects of HHV-6 infection on cytokine synthesis. Since monocytes/macrophages play a major role in the activation of the immune system, we therefore thought it important to first study the effects of HHV-6 on monocyte-derived cytokines, namely interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α); these molecules are potent immunoregulatory proteins, secreted following cell activation (for a review, see references 4, 13, and 21). IL-1β and TNF-α share many biological activities: pyrogenicity, activation of T lymphocytes, stimulation of fibroblast proliferation, and neutrophil activation, to name a few (4, 13). Furthermore, TNF serves as an autocrine tumor growth factor for chronic B-cell malignancies (10). Some of the biological attributes of IL-6 include stimulation of cytotoxic T-cell differentiation together with IL-2 or gamma interferon, growth factor activity for peripheral T lymphocytes, and stimulation of differentiation and antibody production by B lymphocytes (21). Besides having pleiotropic effects on the immune system, these three molecules are involved in inflammatory processes (4, 13, 21). For example, high levels of IL-1β, IL-6, and TNF-α have been found in synovial fluids of patients suffering from rheumatoid arthritis (30, 33, 34). Evaluation of the ability of HHV-6 to induce the secretion of these cytokines following an in vitro infection would give new insights into the pathogenic role of this virus. To our knowledge, very little is known about immunological defense mechanisms against HHV-6. Kikuta et al. (20) demonstrated that HHV-6 induces the secretion of alpha interferon primarily in monocytic and non-T cells. Here we report that HHV-6 induces a rapid and strong IL-1β and TNF-α synthesis in peripheral blood mononuclear cell (PBMC) cultures; interestingly, IL-6 production is unaffected by the presence of the virus. Our data suggest that this cytokine-inducing activity of HHV-6 is associated with a structural viral protein.

Preparation of HHV-6 and PBMC cultures. PBMCs were

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obtained from healthy donors after centrifugation of heparinized venous blood over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients and were cultured as previously described (17).

The HHV-6 used in this study (GS strain [28]) was propagated in HSB-2 cells. HSB-2 is an established human T-cell line which does not require IL-2 or phytohemagglutinin stimulation for its growth or infection with HHV-6. For HHV-6 infection, HSB-2 cells were first incubated with the virus for 2 h at 37°C and then with RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS). The cell-free supernatant from this culture showing evidence of >85% infection (as measured by indirect immunofluorescence assay with human HHV-6 antibody-positive serum and mouse monoclonal antibody to a GS isolate of HHV-6) was filtered through a 0.45-μm-pore-size filter and concentrated by high-speed centrifugation to 100× as previously described (1). The concentrated virus preparation had a titer of 10^5 50% tissue culture infective doses ($TCID_{50}$)/ml. Viral preparations and culture medium, tested for the presence of endotoxin by the limulus amebocyte assay (Sigma, St. Louis, Mo.) were found to contain <10 pg of endotoxin per ml.

PBMCs (10^6/ml) were treated with supernatant from mock-infected HSB-2 culture, HHV-6 (10^3 $TCID_{50}$/ml), heat-inactivated HHV-6 (1 h, 56°C), UV-irradiated HHV-6 (1 h, 265 nm), or lipopolysaccharide (LPS; 1 μg) (Escherichia coli serotype O26:B6; Sigma). Cells treated with phosphonoacetic acid (PAA; Sigma) were incubated with 200 μg of PAA per ml for 1 h prior to virus infection; the antiviral activity of the PAA preparation used was first confirmed by its inhibitory effect on HHV-6 replication, as previously described (12). To ascertain that the effects observed were specific to HHV-6, viral preparations were preincubated with known HHV-6 antibody-positive and antibody-negative sera for 1 h before infection. The antibody-positive serum used was from a patient who had acute lymphocytic leukemia and from whom the GS isolate of HHV-6 was obtained (28). GS serum had an immunoglobulin G antibody titer to HHV-6 of 320, as measured by immunofluorescence assay. This serum, at a dilution of 1:20, neutralized 1,000 $TCID_{50}$ of HHV-6. The serum with gold labelling binds to the surface of HHV-6. In control experiments we found that serum samples adsorbed with Epstein-Barr virus, human cytomegalovirus, and herpes simplex virus type 1 lost their neutralizing activities to these viruses but still retained activities to HHV-6.

The cultures were incubated at 37°C and 5% CO_2, and the supernatants were collected at the times indicated in the figure legends. Cytokine levels (IL-1β, IL-6, and TNF-α) were measured in each supernatant with enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's technical guidelines (R & D Systems, Minneapolis, Minn.).

RNA preparation and PCR amplification. mRNA levels were studied by using a quantitative reverse transcriptase-polymerase chain reaction (PCR) procedure (11). In all cases, cells were lysed at 4, 6, and 12 h posttreatment and total RNA was isolated by using a modified guanidium isothiocyanate procedure (7), including a treatment with 3 U of RQ1 DNase (Promega, Madison, Wis.) for 30 min at 37°C. The details concerning the reverse transcriptase-PCR protocols were as previously described (11). IL-1β primer A is located at bp 21 to 1 (5'-ATGGCAGAAGTACCTGAGCTC-3'), and IL-1β primer B is located at bp 540 to 520 (5'-TTCCTTGAGG AAGGCCAC-3'). The full-length am-

plified fragment has 540 bp. TNF-α primer A is located at bp 2199 to 2175 (5'-CCCTCAAGCTGAGGGGCAGCTCCAG-3'), and the primer B is located at bp 2611 to 2587 (5'-GGGCAATGATCCCAAAGTAGACCTG-3') of the fourth TNF-α exon. The total length of the amplified sequence is 412 bp. IL-6 primer A is located at bp 4144 to 4124 (5'-CAAAGAATCTAGATGCAATAA-3'), and IL-6 primer B is located at bp 4320 to 4299 (5'-GCCCATTAACAACAA CAATCTG-3'). Amplification of the region generates a 201-bp fragment. The 195-bp glyceraldehyde 3-phosphate dehydrogenase (GAPDH) product was amplified with primer A (5'-GGTACCTCTTCCGACCCC-3') located at bp 389 to 372 and primer B (5'-GTTTCAACAGTACCTACTGG-3') located at bp 566 to 547, which were derived from a full-length GAPDH cDNA (3). The GAPDH served as an internal RNA control. The concentration of RNA among the various samples was corrected by calculating the optical density values of IL-1β, TNF-α, and IL-6 bands and the optical density values of the GAPDH bands. In order to calculate the fold increase in RNA transcripts, the ratio obtained for stimulated cells was compared with the ratio for unstimulated cells. Constant amplification in reaction tubes was assessed by coamplifying exogenous DNA (SV_2CAT) in some samples (not shown). Furthermore, all reactions were carried out in conditions that were previously described (11), in which amplification is linear.

Induction of IL-1β and TNF-α by HHV-6. Twelve hours after infection, maximal IL-1β gene transcription was observed. Approximately fourfold induction of IL-1β RNA was detected following infection compared with that in untreated cells (Fig. 1A, lanes 1 and 3). LPS added to PBMCs induced a sevenfold increase in IL-1β transcript levels (Fig. 1A, lane 2). When IL-1β protein levels were measured in culture supernatants, we observed that maximal release of IL-1β (2,500 pg/ml) occurred within the first 48 h of infection and gradually decreased on the following days (Fig. 1B). Compared with that in uninfected cells (600 pg/ml), a fourfold induction in protein secretion was observed (Fig. 1B). The results obtained clearly indicate that HHV-6 is a potent inducer of IL-1β. HHV-6 had also an enhancing effect on TNF-α gene expression. Maximal TNF-α gene transcription was observed at 6 h postinfection. HHV-6 induced a twofold increase in TNF-α mRNA compared with that in uninfected cells (Fig. 2A, lanes 1 and 2). LPS (control) induced a 2.5-fold increase in gene transcripts (lane 3). Maximal TNF-α protein release (1,225 pg/ml) was registered 24 h following infection and decreased over the next 4 days (Fig. 2B). Uninfected cells produced 8.5 times less TNF-α (140 pg/ml) than infected ones (1,225 pg/ml) (Fig. 2B).

To demonstrate that cytokines are induced by HHV-6 and not by other factors (e.g., endotoxin), we preincubated the virus with known HHV-6 antibody-positive and -negative sera and tested the ability of this virus preparation to stimulate cytokine release. Forty-eight hours after treatment with neutralized HHV-6, IL-1β and TNF-α levels were found to be similar to those in uninfected PBMCs (Fig. 1C and 2C). The HHV-6 antibody-negative serum had no effect on HHV-6 capacity to induce cytokines (Fig. 1C and 2C). Furthermore, no detectable levels of IL-2 (a lymphokine mainly secreted by CD4$^+$ T lymphocytes which is known to also be a target for HHV-6) could be measured in supernatants from HHV-6-infected PBMCs. These observations and the notion that HHV-6 can induce TNF-α within 6 h strongly suggest that the cytokine induction observed is specifically attributable to HHV-6. Next, the ability of heat-inactivated
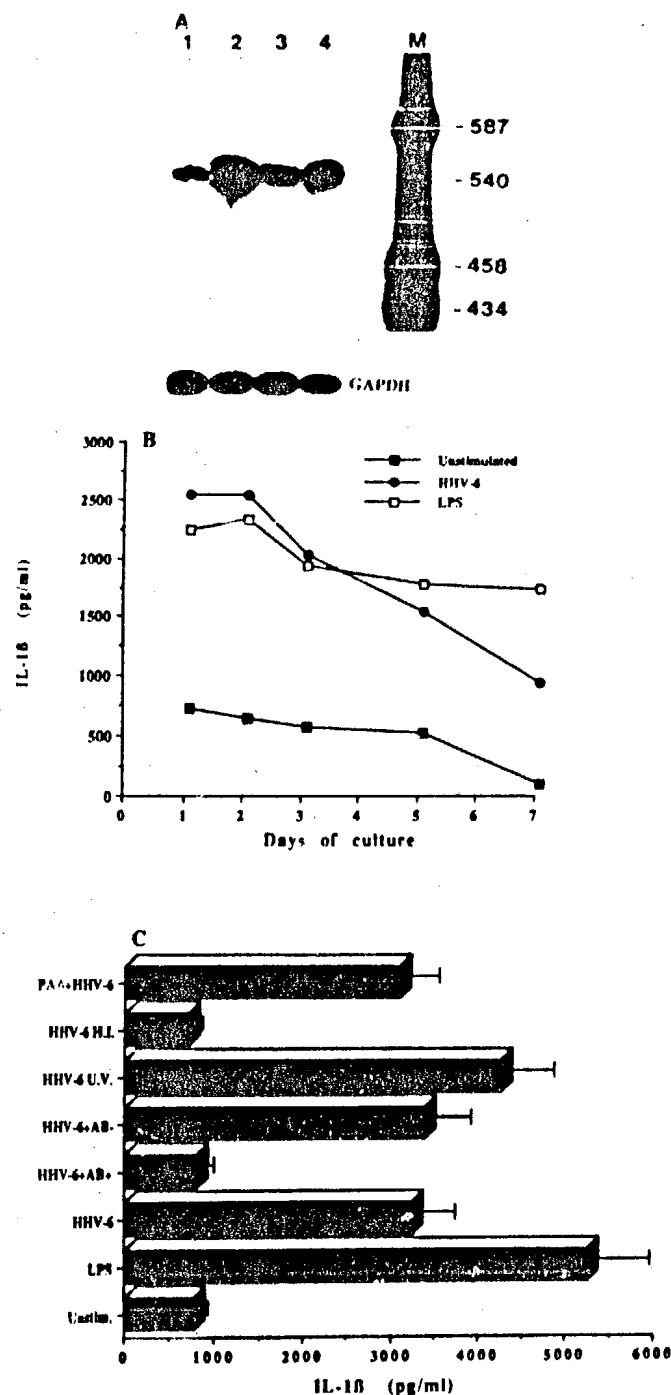
FIG. 1. Analysis of IL-1β gene expression and protein release after HHV-6 infection. (A) Detection of increased IL-1β RNA levels in HHV-6-infected PBMCs by PCR amplification. Total RNA (5 μg) from PBMCs was analyzed on a 4.5% polyacrylamide gel for IL-1β-specific RNA by mRNA phenotyping at 12 h after HHV-6 infection (maximal induction). Lane 1, RNA from untreated PBMCs; lane 2, RNA from LPS-treated (1 μg/ml) cells; lane 3, RNA from HHV-6 infected cells; lane 4, positive PCR control consisting of RNA from U937 cells pretreated with alpha interferon (250 U/ml) for 4 h and then infected with Sendai virus (500 hemagglutinating units per ml). M, Marker plasmid pAT153 cleaved with HaeIII and end labelled. The lower panel represents the GAPDH internal control for each sample. (B) Kinetics of IL-1β production by PBMCs following an HHV-6 infection. PBMCs (10⁶/ml) were exposed to mock-infected culture medium, HHV-6 (10¹ TCID₅₀/ml), or LPS (1 μg/ml). Supernatants were collected at days 1, 2, 3, 5, and 7 and frozen at −70°C. IL-1β production was measured with an ELISA kit. Datum points represent the results with a single donor. The experiment, repeated with PBMCs of three different donors,

and UV-irradiated HHV-6 to stimulate IL-1β and TNF-α synthesis was examined. Heat-inactivated HHV-6 was unable to trigger IL-1β and TNF-α production, whereas UV-irradiated HHV-6 retained the full cytokine-inducing potential of the native virion (Fig. 1C and 2C). This is in agreement with the results of Clouse et al. (9), who found that heat-inactivated HHV-6 was unable to induce monokine secretion. In contrast, heat inactivation of human cytomegalovirus, another herpesvirus, does not affect monokine induction in the same cells (9). To confirm that viral replication is not required to induce IL-1β and TNF-α, we performed experiments with PAA, an inhibitor of viral DNA polymerase activity which is known to effectively inhibit HHV-6 replication (12). Acyclovir was not used because of its poor efficacy against HHV-6 (12). When PBMCs were treated with PAA and subsequently infected with HHV-6, IL-1β and TNF-α syntheses were comparable to those observed in PAA-untreated but HHV-6-infected PBMCs (Fig. 1C and 2C). PAA alone had no effect on cytokine synthesis (not shown). These results support the hypothesis that a thermolabile viral structural protein(s) or conformationally intact virion is responsible for IL-1β and TNF-α induction.

HHV-6 infection and IL-6 production. The same type of experiment was conducted to evaluate the ability of HHV-6 to induce IL-6, a third monocyte/macrophage-derived cytokine. Total RNA was isolated at 4 (Fig. 3A), 6, and 12 h (not shown) posttreatment. Results indicate that HHV-6 has no IL-6-inducing potential compared with uninfected cells (lanes 1 and 3). mRNA levels from infected cells were approximately similar (1.7-fold induction) to levels in noninfected PBMCs, whereas LPS-induced IL-6 transcripts peaked at 4 h with a 4-fold induction (lane 2). IL-6 levels measured in culture supernatants confirm these findings at the protein level. No difference in IL-6 production between untreated cells and HHV-6-infected cells for 7 days (postinfection) (Fig. 3B) was detected. Preincubation of HHV-6 with antibody-positive and -negative sera had no effect on IL-6 secretion (Fig. 3C). Similarly, heat inactivation of the virus had no effect on IL-6 synthesis (Fig. 3C). Although infectious HHV-6 had no stimulatory effect on IL-6 production, UV-irradiated HHV-6 and PAA-treated PBMCs were found to produce slightly more IL-6 than untreated or infected cells (Fig. 3C). This might indicate that viral DNA or viral replication can prevent IL-6 induction. Studies are under way to evaluate how viral replication might affect IL-6 synthesis.

Very little is known about the effects of cytokines on HHV-6. One study demonstrated that exogenously added IL-2 had inhibitory properties on HHV-6 replication in human thymocytes (27). The fact that HHV-6 does not induce IL-6 may represent a strategy by the virus to partly evade immune surveillance. As with the human immunode-

always gave a similar pattern of IL-1β production. (C) Cells (10⁶/ml) were exposed to mock-infected culture medium (Unstim.), LPS (1 μg/ml), HHV-6 (10¹ TCID₅₀/ml), HHV-6 preincubated with an HHV-6 antibody-positive serum (HHV-6+AB+), HHV-6 preincubated with an HHV-6 antibody-negative serum (HHV-6+AB−), UV-irradiated HHV-6 (HHV-6 U.V.), or heat-inactivated HHV-6 (HHV-6 H.I.) or pretreated with PAA (200 μg/ml) and subsequently infected with HHV-6 (PAA + HHV-6). Supernatants were collected after 48 h and kept frozen at −70°C until used. IL-1β production was measured with an ELISA kit. Each datum point represents the mean ± standard deviation of three separate experiments using PBMCs of a high-IL-1β-producing healthy donor.
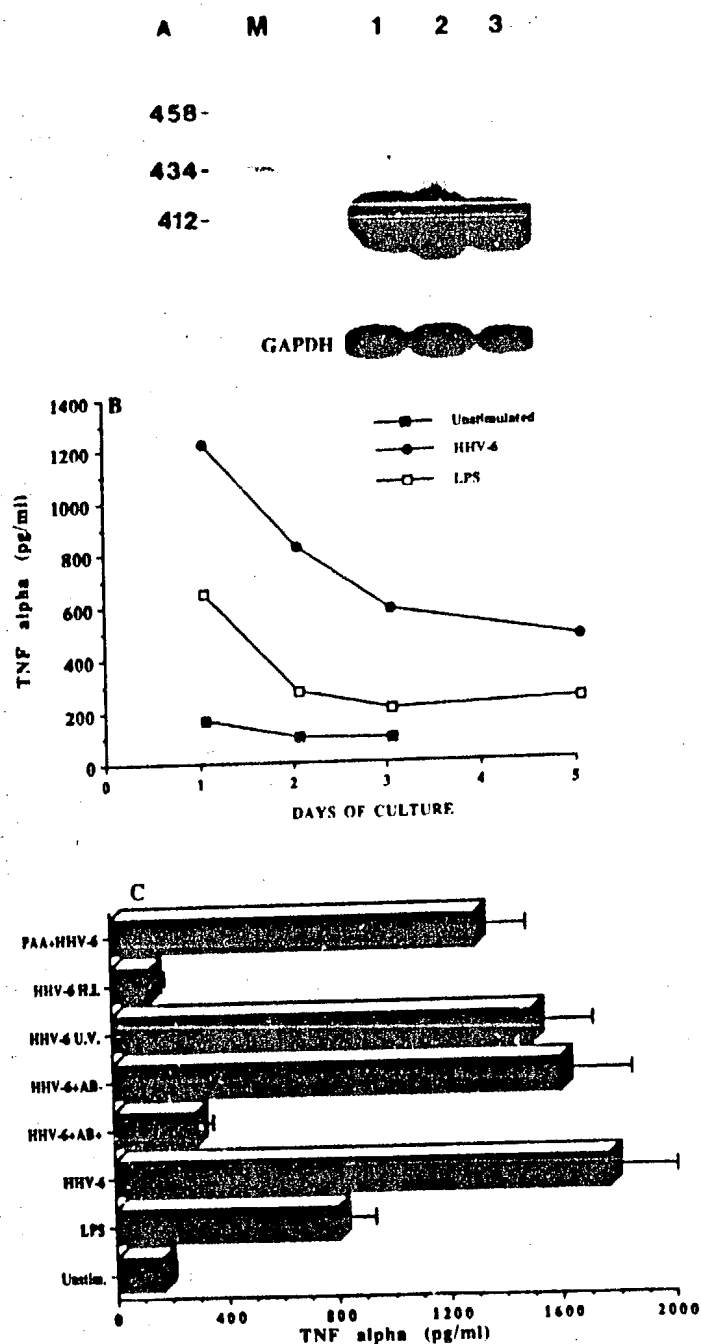
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FIG. 2. Analysis of TNF-α gene expression and protein release after HHV-6 infection. (A) Detection of increased TNF-α RNA levels in HHV-6-infected PBMCs by PCR amplification. mRNA phenotyping was performed 6 h after HHV-6 infection (maximal induction). Lane 1, RNA from untreated PBMCs; lane 2, RNA from HHV-6-infected cells; lane 3, RNA from LPS-stimulated (1 μg/ml) cells. The positions of the marker fragments (pAT153 × HaeIII) in lane M are indicated on the left. The lower panel corresponds to the GAPDH control for each sample. (B) Kinetics of TNF-α production by PBMCs following an HHV-6 infection. PBMCs were treated as described in the legend to Fig. 1B. TNF-α production was measured with an ELISA kit. Datum points represent the results with a single donor. The experiment, repeated with PBMCs of three different donors, always gave a similar pattern of TNF-α production. (C) Cells were prepared and treated as described in the legend to Fig. 1C. TNF-α production was measured with an ELISA kit. Each datum point represents the mean ± standard deviation of three separate experiments with PBMCs of a high-TNF-α-producing healthy donor.
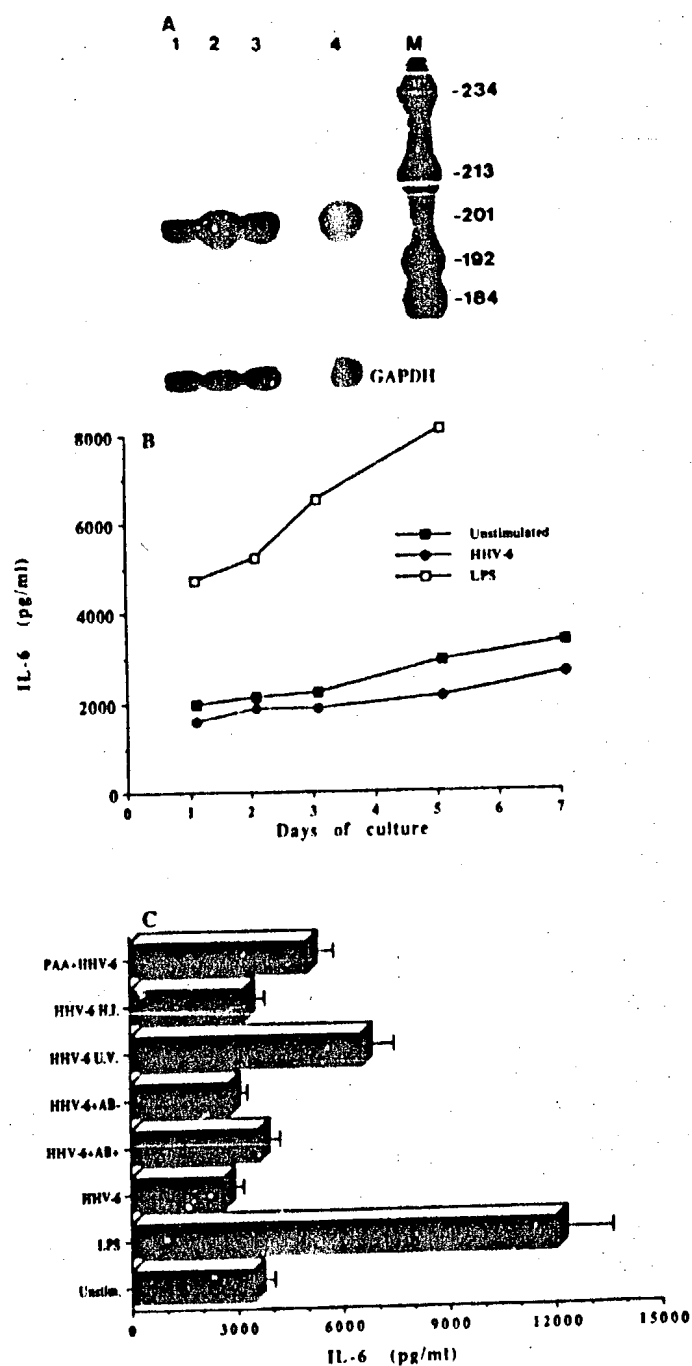
FIG. 3. Analysis of IL-6 gene transcript and secreted gene product following an HHV-6 infection. (A) Total RNA (5 μg) from PBMCs was analyzed for IL-6-specific RNA by PCR amplification. Analysis was performed after 4, 6, and 8 h posttreatment. Maximal IL-6 transcription was observed at 4 h, after which diminished IL-6 transcripts are observed (not shown). Lanes are described in the legend to Fig. 1A. (B) Kinetics of IL-6 production by PBMCs following an HHV-6 infection. PBMCs were treated as described in the legend to Fig. 1B. IL-6 production was measured with an ELISA kit. Datum points represent the results with a single donor. The experiment, repeated with PBMCs of three different donors, always gave a similar pattern of IL-6 production. (C) Cells were treated as described in the legend to Fig. 1C. IL-6 production was measured with an ELISA kit. Each datum point represents the mean ± standard deviation of three separate experiments using PBMCs of a high-IL-6-producing healthy donor.

ficiency virus (14), IL-1β and TNF-α might have a stimulatory effect on HHV-6 gene expression.

The tropism and infectivity of HHV-6 for cells of the monocyte/macrophage lineage has been demonstrated by Levy et al. (24), who reported that HHV-6 (SF strain) can infect monocytes. Similar results were observed in our laboratory with the GS strain (unpublished data). Infected monocytes rarely produce virus, suggesting that HHV-6 remains in a latent state. The fact that HHV-6 reactivation in cases of chronic fatigue syndrome and lupus erythematosus and in otherwise immunocompromised patients has been observed suggests that HHV-6 plays a role in the course of these diseases (22). Furthermore, the TNF-α-inducing potential of HHV-6 is of particular interest in AIDS patients since this monokine is known to enhance human immunodeficiency virus type 1 expression (14).

Finally, it is noteworthy that the effects of HHV-6 infection on TNF-α and IL-6 we described above are quite in contrast to those observed with Epstein-Barr virus, also a human herpesvirus. Interestingly, Epstein-Barr virus was found to inhibit TNF-α synthesis (16) on one hand and to stimulate IL-6 production on the other (unpublished data).

Taken together, our results indicate that HHV-6 stimulates IL-1β and TNF-α productions but has no effect on IL-6 synthesis. These observations reinforce the notion that HHV-6 can infect cells of myeloid lineage and may contribute to the understanding of the pathogenicity of a virus that has a marked tropism for cells of the immune system.

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